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1 Optimising the composition of irrigation fluid to reduce the potency of *S. aureus* α -toxin:
2 potential role in the treatment of septic arthritis.
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22 Running Head: Optimising cartilage irrigation fluid
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25 pressure.
26
27

Abstract

Objective: Septic arthritis is commonly caused by *Staphylococcus aureus* and is a medical emergency requiring antibiotics and joint irrigation. The bacteria produce α -toxin causing rapid cartilage cell (*chondrocyte*) death. Saline (0.9%NaCl) lavage is normally used to remove bacteria and toxins, however, its composition might be sub-optimal to suppress the lethal effects of α -toxin. We utilised rabbit erythrocyte haemolysis as a sensitive, biologically-relevant assay of α -toxin levels to determine if changes to osmolarity, temperature, pH, and divalent cation (Mg^{2+} , Ca^{2+}) concentration were protective.

Design: Erythrocytes were incubated in the various conditions and then exposed to α -toxin ('chronic' challenge) or incubated with α -toxin and then exposed to experimental conditions ('acute' challenge).

Results: Raising osmolarity from 300mOsm (0.9%NaCl) to 400, 600 or 900mOsm (sucrose addition) when applied chronically, significantly reduced haemolysis linearly. As an acute challenge, osmotic protection was significant and similar over 400-900mOsm. Reducing temperature chronically from 37°C to 25°C and 4°C significantly reduced haemolysis, however, when applied as an acute challenge although significant, was less marked. Divalent cations (Mg^{2+} , Ca^{2+} at 5mM) reduced haemolysis. Varying pH (6.5, 7.2, 8.0) applied chronically marginally reduced haemolysis. The optimised saline (0.9%NaCl;900mOsm with sucrose), 5mM $MgCl_2$, (37°C) rapidly and significantly reduced haemolysis compared to saline and Hank's buffered saline solution (HBSS) applied either chronically or acutely.

Conclusions: These results on the effect of *S. aureus* α -toxin on erythrocytes showed that optimising saline could markedly reduce the potency of *S. aureus* α -toxin. Such modifications to saline could be of benefit during joint irrigation for septic arthritis.

Introduction

Septic arthritis, a destructive joint disease leading to permanent cartilage damage and disability, affects all ages with an incidence in Western Europe of 4-10 cases/100,000 persons/yr^{1,2}. *Staphylococcus aureus* (*S. aureus*) accounts for 40-70% of all cases of septic arthritis^{1,3,4} and the incidence is rising due to factors including an ageing population, increased use of immunosuppressive agents, musculoskeletal prosthetics and surgical procedures⁵. Management of septic arthritis is to rapidly eliminate the bacteria and associated toxins through intravenous antibiotics and copious joint irrigation⁵. Despite eradication of the bacteria, the damage caused to articular cartilage persists in >50% of cases and may lead to osteoarthritis (OA)^{1,2,6}.

S. aureus produces an array of toxins including exotoxins (including alpha (α), beta (β), gamma (γ) and delta (δ) haemolysin)⁷ which are potent virulence factors⁸⁻¹¹. Recent work using *in vitro* bovine cartilage and an *in vivo* murine model of septic arthritis with isogenic mutants of *S. aureus*, has identified α -toxin as the primary agent causing the rapid death of cartilage cells (chondrocytes)¹²⁻¹⁴. Chondrocytes are the only cell type capable of maintaining the tissue's resilience through the turnover of extracellular matrix proteins and their loss, through the action of α -toxin, will result in cartilage degradation. *S. aureus* α -toxin also has longer-term damaging effects on chondrocytes, for example, it may increase expression of catabolic factors including matrix metalloproteinases (MMPs) and inducible NO synthase (iNOS) leading to deleterious changes to cartilage metabolism¹⁵⁻¹⁸. While there has been considerable attention given to the development of antibacterials for treating *S. aureus* infection, the protection of chondrocytes against the deleterious effects of α -toxin has not been as intensively investigated.

S. aureus α -toxin binds to the A Disintegrin And Metalloproteinase 10 (ADAM10) receptor present on animal and human articular chondrocytes and rabbit erythrocytes¹⁹⁻²¹. This leads to the formation of a heptameric pore and rapid influx of Na⁺ and water, causing cell swelling and lysis, leading to the release of intracellular components resulting in inflammation²². Rabbit erythrocytes show only low sensitivity to other haemolysins²³ (in contrast to human erythrocytes²⁴) and are therefore an extremely flexible and sensitive model system for studying the interaction between this α -toxin and cell lysis²⁴. Additionally, the release of haemoglobin can easily be measured spectrophotometrically, allowing the dynamic effects of biologically-relevant activity of α -toxin on cell viability to be assessed²⁴.

The fluid used for joint irrigation is normally isotonic saline (0.9%NaCl;300mOsm) which is hypo-osmotic compared to normal synovial fluid (400mOsm)²⁵. Previous work has shown that the sensitivity of chondrocytes to other forms of injury may be markedly reduced when the osmolarity of isotonic saline or culture medium (typically 300mOsm) is increased^{26,27}. This raised the possibility that the saline currently used for irrigation might be sub-optimal and that altering some of its properties might reduce the injurious effects of α -toxin and thus be chondroprotective against α -toxin. Accordingly, we have tested the effects of osmolarity (300, 400, 600, 900mOsm),

101 temperature (4°C, 25°C, 37°C), divalent cations (Ca²⁺ and Mg²⁺) and pH (6.5, 7.2, 8.0) on the
102 potency of *S. aureus* α-toxin using the sensitive rabbit erythrocyte haemolysis assay. The aim of
103 this study therefore was to determine if these relatively simple alterations to the properties of
104 standard saline could reduce the damaging effect of *S. aureus* α-toxin.

Materials and Methods.

(a) *Biological materials, tissue culture, reagents.* Saline (0.9%;300mOsm) used clinically for irrigation was obtained from Baxter Healthcare Ltd., Norfolk, UK. Hank's buffered saline solution (HBSS;300mOsm) was purchased from Invitrogen Ltd., Paisley, UK. HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer was obtained from Sigma-Aldrich Chemical Co., Gillingham, UK. TSA (tryptone soya agar), TSB (tryptone soya broth) and skimmed milk were obtained from Oxoid Ltd., Basingstoke, UK.

(b) *Rabbit red blood cells.* The rabbit red blood cell (RBC) haemolysis assay was used to semi-quantitatively determine biologically relevant α -toxin activity²⁴. Fresh, heparinised RBCs from Orygen Ltd., Penicuik, UK, were prepared at ~5% haematocrit in saline (0.9% NaCl) and kept at 5°C until required. Fresh blood was obtained weekly and was suitable for up to 5 days of experimentation after receipt.

(c) *Preparation of bacterial pellets and supernatant samples.* *S. aureus* strain 8325-4 was kindly provided by Prof. T.J. Foster and stored at -80°C in 10%v/w skimmed milk. This strain is a well-characterised prophage-cured derivative of strain NCTC8325 that produces large amounts of α -toxin²⁸ and has comparable potency to clinical strains of *S. aureus* in terms of chondrocyte-damaging potential¹². When required, bacteria were thawed and streaked onto TSA plates and cultured (24hrs;37°C). TSB plates containing 2 μ g/ml tetracycline were then prepared and inoculated with several single bacterial colonies from the TSA plate and incubated (24hr;37°C) with shaking. From this culture, serial dilutions were performed in saline to 10⁻⁶, spread on TSA plates and incubated (24hr;37°C). The number of Colony Forming Units/ml (CFU/ml) in TSB was typically ~1x10⁹ CFU/ml. α -toxin-containing supernatants were obtained by centrifugation (800xg;10min) of the TSB cultures which were then filter-sterilised and stored (4°C) until required which was within one week. To establish an appropriate time-course at the beginning of a week's experiments, the sensitivity of rabbit RBCs to α -toxin were assessed by adding a small volume of toxin to a 5% RBC suspension and incubating at 37°C for 60mins. Samples were taken every 10mins, centrifuged (10,000xg;10secs) and haemolysis determined by the absorbance of haemoglobin at 540nm (Abs₅₄₀) on a Nanodrop spectrophotometer (ThermoFisher Scientific, Waltham, USA). Maximum (100%) haemolysis was determined by freeze/thawing a sample of the RBC suspension. Percent haemolysis (%H) was then calculated ((Abs₅₄₀ of sample – Abs₅₄₀ of negative control)/(Abs₅₄₀ of 100% haemolysis – Abs₅₄₀ of negative control) x100%) to give a measure of α -toxin activity. The sensitivity of erythrocyte samples to α -toxin and the amount and potency of α -toxin produced from each *S. aureus* culture, were variable. Rabbit erythrocytes which were relatively insensitive to toxin requiring >1hr of incubation before haemolysis was detectable, were not studied further. A rabbit blood sample which produced ~50% haemolysis after about 15mins was considered acceptable for experimentation.

(d) *Chronic or acute exposure of α -toxin-treated erythrocytes to various solutions and temperatures.* *Chronic exposure:* Suspensions of rabbit erythrocytes were initially exposed to the

149 experimental conditions of osmolarity, temperature, pH or divalent cations for 10mins before an
150 aliquot of α -toxin was added, the cell suspension mixed quickly, and the time course of %
151 haemolysis (%H) measurements commenced. *Acute exposure:* The α -toxin treated erythrocyte
152 suspensions were initially incubated under control conditions and haemoglobin release measured
153 until this reached 20-30% haemolysis. The cell suspension was then challenged with the various
154 experimental conditions, and the extent of haemolysis determined until the end of the time course.
155 The rate of change in % haemolysis/10mins for the chronic challenge was measured over 10-
156 20mins and for the acute challenge, the time course was measured over 20-30mins after the start
157 of the experiment. Data were shown as the change in % haemolysis (%H)/10mins. For the control
158 condition in the 'acute exposure' experiments for osmolarity and divalent cations, an identical
159 volume of saline was added at the same time point to correct for α -toxin dilution. For the pH
160 experiments, HEPES (10mM) was present and pH adjusted using HCl or NaOH. For some
161 experiments, erythrocytes were suspended in Hank's balanced salt solution (HBSS;pH 7.2).
162 *(e) Data analysis and statistics.* Data are shown as means \pm standard error of the mean (s.e.m.) for
163 N independent experiments and n replicates for each experiment ($N(n)$), and analysed using
164 GraphPad Prism Ver. 7.0b (GraphPad, San Diego, USA). Non-parametric t-tests and ANOVAs
165 (Analysis of Variance) were performed as indicated and significant differences accepted when
166 $P < 0.05$.

167

Results

(a) *Suppression of α -toxin damage by raising saline osmolarity.* To assess the effects of raising saline osmolarity (300mOsm) above that of synovial fluid (i.e. ≥ 400 mOsm) on the damaging effect of α -toxin on rabbit erythrocytes, two types of experiments were performed: (i) *chronic challenge* – where erythrocytes were exposed to the various osmotic conditions including α -toxin throughout (Fig. 1A), or (ii) *acute challenge* – where erythrocytes were exposed to α -toxin until approx. 20-30% haemolysis had occurred, before the hyper-osmotic challenge was delivered (Fig. 1B). For the chronic challenge, by the end of the time-course, control percent haemolysis (%H) (300mOsm; ~93%) was significantly greater than 400mOsm (72%; $P=0.0008$), 600mOsm (47%; $P<0.0001$) and 900mOsm (21%; $P<0.0001$) (Fig. 1A(i)). An inverse linear relationship was evident between osmolarity and %H induced by α -toxin ($R^2=0.9626$; $P=0.0189$; (Fig. 1A(ii)). The change in % haemolysis/10mins was calculated after toxin exposure and was 3.9% for the control and although reduced at 400mOsm, was not significant (2.2%; $P=0.506$). However a reduction was observed at 600mOsm (0.7%; $P<0.0001$) and 900mOsm (0.1%; $P<0.0001$; (Fig. 1A(iii)).

When osmolarity was raised >300 mOsm, approximately 10mins after α -toxin addition ('acute challenge') there was rapid and almost complete protection of erythrocytes (Fig. 1B(i)). There was a significant decrease in haemolysis between the control (300mOsm; 82%) and hyperosmotic solutions (44%, 41%, 41% for 400, 600, 900mOsm respectively) by the end of the experiment ($P<0.0001$ for all osmolarities compared to 300mOsm). Interestingly, in contrast to the chronic challenge, the effects of 400mOsm and greater were not significantly different when compared to each other ($P=0.327$; Fig. 1B(ii)). The rates of change in %H were significantly decreased for osmolarities of ≥ 400 mOsm (Fig. 1B(iii)). Raising osmolarity using sucrose was thus strongly protective particularly after α -toxin damage to erythrocytes had been initiated.

The osmotic protection conferred by sucrose was compared with that of a different osmolyte (NaCl) to the same osmotic pressure. At 40mins, NaCl (600mOsm) reduced %H from the control (300mOsm) value of $66\pm 6\%$ to $56\pm 8\%$, whereas with sucrose this was decreased further to $20\pm 3\%$. In additional experiments at the same time point, when the osmolarity was raised to 900mOsm, the %H for the control was $68\pm 8\%$, and reduced to $21\pm 7\%$ in the presence of NaCl but was only $4\pm 2\%$ with sucrose (data are means \pm s.d., $N=2$). Although NaCl protected erythrocytes against α -toxin, it appeared less effective when present at the same osmolarity as sucrose.

(b) *Reducing temperature of saline conferred protection against α -toxin-induced haemolysis.* RBC suspensions were incubated for 10mins at the various temperatures, treated with α -toxin and then haemolysis determined. The chronic exposure to reduced temperature protected erythrocytes treated with α -toxin (Fig. 2A(i)). After 60mins at 25°C, the %H was 48% and significantly less compared to 37°C (75%; $P=0.0056$). However, when the α -toxin treated red blood cells were incubated at 4°C, haemolysis was virtually abolished over the time course studied ($P<0.0001$; Fig.

206 2A(i)). An inverse linear regression was observed between decreasing temperature and
 207 haemolysis ($R^2=1.00$; $P=0.002$) (Fig. 2A(ii)). The rate of change in haemolysis (%H/10mins) for 4°C,
 208 25°C, and 37°C was 0.02%, 2.0%, and 2.6% respectively (4°C vs. 37°C, $P=0.0115$; 4°C vs. 25°C,
 209 $P=0.0451$) (Fig. 2A(iii)). When temperature was changed quickly during the time course
 210 ('temperature switch protocol'), no significant difference in haemolysis was observed between 37°C
 211 and 25°C until the 50min time-point (71% vs. 66%; $P=0.0232$; Fig. 2B(i)). A significant difference
 212 was also found between 37°C and 4°C after 40mins (65% vs. 59%; $P=0.001$). Although a linear
 213 relationship was evident, there was no significant deviation from a gradient of zero
 214 ($R^2=0.979$; $P=0.0923$; Fig. 2B(iii)). Therefore, reducing temperature was more protective before α -
 215 toxin addition. However, after its addition, reducing the temperature from 37°C to 4°C reduced
 216 haemolysis by ~10% (Fig. 2B(i)).

217 (c) *Protective effect of divalent cations against α -toxin induced erythrocyte haemolysis.* For the
 218 chronic exposure experiments, divalent cations (Ca^{2+} or Mg^{2+} at 5mM) produced a modest
 219 decrease in haemolysis with significant protection observed as early as 10mins with CaCl_2
 220 ($P=0.0149$) and 20mins with MgCl_2 ($P<0.0001$; Fig. 3A(i)). This was maintained throughout the
 221 exposure protocol. By the end of the experiment, lower haemolysis levels resulted from cell
 222 suspensions containing Ca^{2+} (58.8%, $P=0.0063$) or Mg^{2+} (60.7%, $P=0.0231$) compared to the
 223 control (71.7%; Fig. 3A(i)). The protective effects of the divalent cations were indistinguishable
 224 ($P>0.99$; Fig. 3A(i, ii)). The addition of Mg^{2+} after α -toxin exposure ('acute exposure') gave
 225 significant protection ($P=0.0002$) against haemolysis, however, no difference was recorded with
 226 Ca^{2+} ($P=0.12$) at 60mins (Fig. 3B(i)). Furthermore, the rate of change showed no differences
 227 between the control and divalent ions ($P>0.05$; Fig. 3B(ii)). Thus, divalent ions provided only minor
 228 protection against haemolysis induced by α -toxin.

229 (d) *Influence of pH on α -toxin induced erythrocyte haemolysis.* Altering saline pH might provide
 230 some protection against the damaging effects of α -toxin. A significant decrease in %H occurred
 231 between pH 6.5 and 8.0 (two-way ANOVA; $P=0.0015$) at 60mins (Fig. 4(i)). However, there were no
 232 significant differences between normal pH (pH 7.2) and pH 6.5 ($P=0.1995$) or pH 8.0 ($P=0.0892$).
 233 The data points fitted a linear regression but the slope was not significantly different from zero
 234 ($R^2=0.9685$, $P=0.1136$; Fig. 4(ii)). The rate of change of %H between 10-20mins of toxin exposure
 235 showed little variation across the pH values ($P>0.99$) (Fig. 4(ii)) suggesting that increasing saline
 236 pH may only offer marginal protection.

237 (e) *Effect of optimised saline on α -toxin-induced erythrocyte haemolysis.* On the basis of the
 238 previous results, an optimised saline solution (0.9%NaCl, 900mOsm, 5mM MgCl_2 , 37°C) was
 239 prepared. This was compared to normal saline (0.9%NaCl) and HBSS (which contains Ca^{2+}) in its
 240 ability to protect erythrocytes against α -toxin (Fig. 5A(i)). When the red blood cells were pre-
 241 incubated in these solutions for 10mins before addition of α -toxin, the optimised saline abolished
 242 subsequent haemolysis in contrast to both normal saline and HBSS (both $P<0.0001$), where levels
 243 of haemolysis were 78% (normal saline), 63% (HBSS), 4% (optimised saline) at 60mins (Fig.

244 5A(i)). The rate of change of haemolysis was greatest for normal saline (3.3%) followed by HBSS
245 (1.7%) then optimised saline (0.05%;Fig. 5A(ii)). The addition of optimised saline after toxin
246 exposure also significantly suppressed haemolysis compared to the other solutions. With optimised
247 saline, haemolysis showed a small increase from 36% to 46% by the end of the experiment,
248 whereas these levels rose considerably more with the use of normal saline (75%) and HBSS
249 (72%;Fig. 5B(i)). Likewise, the rate of change was much reduced with optimised saline
250 (0.2%H/10mins) compared to normal saline (1.3%H/10mins, $P=0.0374$) and HBSS
251 (1.4%H/10mins, $P=0.03$;Fig. 5B(ii)).

252

253

Discussion

During treatment for *S. aureus* septic arthritis, it is important that the synovial fluid and infected tissues of the joint are rinsed quickly with a benign solution to remove bacteria and associated toxins. Traditionally, saline (0.9% NaCl) is used, however this might be sub-optimal for suppressing α -toxin activity and there may be opportunities for its composition and other properties to be modified to protect chondrocytes. To assess the protective effects of these modified solutions, the release of haemoglobin from rabbit erythrocytes was used as a sensitive and biologically-relevant measure of *S. aureus* α -toxin activity. The results suggested that increased osmolarity, reduced temperature, divalent cations, and to a lesser extent alkaline pH, could significantly reduce the damaging effect of α -toxin suggesting that relatively simple modifications to saline could be of benefit during joint irrigation for septic arthritis.

It could be considered that the rabbit haemolysis assay for determining methods for protecting cells against the damaging effects of α -toxin would not be an appropriate model. However, rabbit erythrocytes contain the *S. aureus* α -toxin receptor ADAM10 which is also present on chondrocytes of animals and normal and degenerate human cartilage¹⁹⁻²¹. The measurement of haemoglobin release following the interaction between α -toxin and erythrocytes provided a dynamic and sensitive assay for the lethal effects of α -toxin, and was highly flexible experimentally and reproducible. While it would be possible test these conditions on cartilage explants, interpreting the results obtained under these various conditions might not as straightforward, as for example access to the receptor in cartilage zones could be delayed and/or restricted. While studies on isolated chondrocytes could be of benefit, the receptor may be damaged or its sensitivity altered as a result of the enzymic treatment of cartilage which is required for release of chondrocytes. Our previous work has demonstrated that *S. aureus* α -toxin is the key damaging agent to chondrocytes in a cartilage model of septic arthritis^{12,14}. Thus, although the rabbit erythrocyte model could be considered a limitation in this study, it nevertheless yielded valuable information about whether protection against α -toxin was possible and identified alterations to the irrigation fluid which could potentially be extended to detailed *in vivo* and clinical studies on *S. aureus* septic arthritis.

Two protocols were used which would broadly correspond to different stages of α -toxin action on the cell membrane which is time-dependent with the binding and pore-formation occurring within 2-3 mins²⁴. For the chronic challenge, erythrocytes were equilibrated with the various conditions (osmolarity, temperature, pH, divalent cations), before toxin was added and the haemolysis time-course commenced. For the acute challenge, the time-course was started by α -toxin addition to the RBC suspension, and when there was 20-30% haemolysis, the erythrocytes were exposed to the experimental conditions. Thus, the chronic exposure would mainly represent the effect of experimental conditions on early steps of toxin action but for the acute exposure, pore formation would be complete and the pathological changes (i.e. increased ion permeability, cell swelling) would be underway with haemolysis following. The acute exposure would be closer to the clinical situation where the majority of cells in the joint would already have been exposed to

292 prevailing levels of α -toxin, and cell injury/death would be proceeding. While the overall effects of
293 chronic and acute challenges of osmolarity, reduced temperature and divalent cation concentration
294 (Figs. 1-4) were similar and gave significant protection, the time courses appeared different.

295 Raising osmolarity prior to toxin addition (chronic osmotic challenge) reduced the rate of
296 haemolysis in a dose-dependent manner (Fig. 1A(i)-(iii)). This suggested that erythrocyte
297 shrinkage could have interfered with early events of toxin action i.e. monomeric α -toxin binding to
298 the cell membrane and pore formation. However studies by Cooper *et al.*²⁴ where an osmolyte
299 (polyethylene glycol) was added after α -toxin addition, suggested that binding and pore formation
300 were unaffected and that pore permeability instead was more sensitive to osmolarity. An acute
301 hyper-osmotic challenge to erythrocytes in which α -toxin pores would already have formed, was
302 rapidly (within 10mins) effective over the range studied (400-900mOsm). However, there was no
303 difference between the osmolarities (Fig. 1B(i)-(iii)). This protective effect may be different
304 compared to the chronic challenge, with the acute hyper-osmotic medium causing rapid
305 erythrocyte shrinkage thereby conferring protection against the cell swelling induced by α -toxin.
306 The raised osmolarity might simply shrink the cells rapidly meaning that it would take longer for the
307 cells to swell to a critical volume. It was noted that NaCl was less effective at protecting
308 erythrocytes compared to sucrose to the same osmolarity. This may be because Na^+ can enter *via*
309 Na^+ channels and $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter²⁹ effectively reducing the osmolarity compared to
310 sucrose. Thus, sucrose would be the preferred osmolyte for the optimised irrigation fluid as it is
311 impermeable and metabolically inert (see²⁶).

312 Pre-incubating erythrocytes at 25°C delayed the damaging action following α -toxin addition.
313 However once established, the rate of increase in haemolysis was the same as for cells
314 equilibrated at a physiological temperature (Fig. 2A(iii)). This suggested that the early steps of pore
315 formation were sensitive to reduced temperature. However, pre-equilibration at 4°C completely
316 protected erythrocytes against α -toxin (Fig. 2A(i)). This may accord with Reichwein *et al.*,³⁰ who
317 demonstrated a temperature-dependent transition from toxin monomers to a functional heptameric
318 pore. They pre-incubated rabbit erythrocytes with α -toxin (0°C;30mins), and then washed and
319 maintained the erythrocytes at either 0°C or 37°C. Enzyme-linked immunosorbent assays (ELISA)
320 showed that there were no α -toxin oligomers on the cell membrane nor any haemolysis at the
321 lower temperature. In contrast, the cells that were incubated at 37°C experienced haemolysis,
322 suggesting that α -toxin binding/pore formation was suppressed at low temperature. This is
323 supported by Freer³¹ who showed that α -toxin binding did not necessarily lead to erythrocyte
324 destruction and that lysis (i.e. functional pore formation) did not occur until temperatures were
325 >12°C. Notably, the effects of reduced temperature in the acute challenge experiments (Fig. 2B(i))
326 were less marked than for the chronic challenge as there was a delay before the inhibition
327 occurred (Fig. 2(A,B)). This could be because the pores had formed, and reduced temperature had
328 little effect on the cation flow and subsequent erythrocyte swelling. Clinically, the acute challenge
329 methodology would represent the situation where irrigation fluid was introduced into the infected

joint during treatment. The toxin would already be present, bound to the cell membranes and acting on chondrocytes and other cells in the joint to cause its damaging effects.

There was a mild but significant protection of erythrocytes when pH was increased from 6.5 to 8.0 (Fig. 4(i)). The pH of 0.9% NaCl should be ~7.0 but the true value often oscillates around pH=5.5 due to varying levels of dissolved CO₂³²). Work by others³³ suggested that acidity converted α -toxin from an amphipathic form into a more hydrophobic molecule, thus accelerating pore formation. It has also been proposed that acidic pH enhanced H⁺ binding to histidine residues on the toxin molecule vital for polymerisation, accelerating pore formation³⁴. There may therefore be benefit to introduce a benign pH buffer to stabilise irrigation fluid pH, perhaps at a slightly alkaline level, to provide some protection against α -toxin and also dampen any elevated pCO₂ levels present in the clinical environment.

Ca²⁺ and Mg²⁺ produced small but significant protection against α -toxin (Fig. 3A,B) with no differences between these ions. Previous studies on Ca²⁺ suggested that it reduced the lateral movement of monomeric α -toxin in the plane of the membrane thereby reducing the rate of pore formation³⁵. Depletion of Ca²⁺ was sufficient to remove the protection suggesting a reversible effect. However, it was unclear if this was Ca²⁺-specific or whether due to the osmolarity of the CaCl₂ which would contribute ~90mOsm³⁵. Apart from the protective effects of divalents on the action of α -toxin on cells, antibacterial roles for Ca²⁺ and Mg²⁺ have been reported³⁶. Raised divalent concentrations disrupted *S. aureus* membranes possibly by forming complexes with cardiolipin which introduced membrane bending and destabilised its integrity. Stationary-phase bacteria, which are resilient against environmental pressures, were subjected to either divalent ion for 40mins and ~60% of the bacterial culture did not survive. A threshold of 10mM Ca²⁺ and 20mM Mg²⁺ to destroy *S. aureus* was established³⁶. This study used concentrations greater than the present work, and therefore it may be interesting to further investigate increasing Ca²⁺ and Mg²⁺ concentrations on *S. aureus* survivability and α -toxin potency.

On the basis of these experiments, we tested an optimised saline applied as either a chronic or an acute challenge and observed substantial protection of erythrocytes against α -toxin (Fig. 5A,B). That there was little difference between normal saline and HBSS suggested that the majority of the protection was due to the raised osmolarity. With the acute challenge (Fig. 5B), the protection was very rapid indicating the quick suppression of the damaging effect of α -toxin which could be considered potentially clinically relevant for joint irrigation. There was still, however, a small increase in %H (Fig. 5B) possibly because further optimisation may be required, and/or there are other toxic elements produced by *S. aureus* which could have a relatively minor damaging effect on rabbit erythrocytes. A modified irrigation solution may also have benefits beyond those of protecting cells against α -toxin. For example, cooled irrigation fluid could offer pain relief and anti-inflammatory effects. A study involving patients who underwent total knee arthroplasty found that saline administered at 4°C alleviated pain, localised swelling, and decreased analgesia intake, as well as improving the quality of post-operative recovery³⁷. Furthermore, a hyper-osmolar irrigation

368 saline, in addition to rapid protection against α -toxin, could be beneficial as Chan and Foster³⁸
369 found that addition of 20mM sucrose in growth media suppressed α -toxin gene (*hla*) expression by
370 ~98% of the control.

371 While the present results were obtained using the rabbit red blood cell model, some caution
372 should be exercised when extrapolating these results to the protection of chondrocytes within the
373 cartilage matrix. Previous studies using a bovine osteochondral explant model have shown that *S.*
374 *aureus* α -toxin can rapidly penetrate the matrix and cause chondrocyte death¹². We have also
375 shown that chondrocyte volume changes very quickly (within mins) following alterations to
376 extracellular osmolarity³⁹. Thus there is the expectation that by raising osmolarity, protection of *in*
377 *situ* chondrocytes against α -toxin should be achieved in the same way this has been demonstrated
378 with rabbit erythrocytes. With these observations in mind and taking the results from the present
379 study together with previous observations, the beneficial effects of modifying the irrigation saline
380 used during joint lavage should be considered in further *in vivo* animal and/or clinical research. The
381 use of a relatively benign, inexpensive, drug-free and rapidly-effective modified saline as part of the
382 normal lavage process is potentially an attractive novel method for limiting the damaging action of
383 *S. aureus* α -toxin during septic arthritis.

384 *S. aureus* infections have been treated with β -lactams (e.g. penicillin) for decades, but the
385 appearance and rapid spread of methicillin-resistance *S. aureus* (MRSA) have all but eliminated
386 these antibacterials for treatment⁴⁰. Non-antibiotic treatment is therefore an area of important
387 research interest since suppressing activity of bacterial toxins either by influencing toxin
388 production, or blocking their action would not only make the bacteria less pathogenic, but may also
389 increase their susceptibility to host immune defence⁴¹. For example, inhibition of *S. aureus*
390 pathogenesis by interfering with the signal transduction pathways for virulence using the RNA III
391 inhibiting peptide has been described⁴². This peptide reduced the pathology and delayed the onset
392 of disease symptoms in models of *S. aureus* infection including septic arthritis⁴². Other methods
393 include an α -toxin antibody⁴³, cyclodextrin-lipid complexes to suppress the damaging effect of *S.*
394 *aureus* α -toxin⁴⁴ and nanoparticle-based α -toxin entrapment to deliver the non-disrupted pore-
395 forming toxin for immune processing⁴⁵. These methods could be particularly important for cells with
396 high levels of the ADAM10 receptor²¹ which would render them particularly sensitive to *S. aureus*
397 α -toxin. In summary, the development of the optimised irrigation saline described here potentially
398 offers a cheap, very rapid (within minutes) and relatively benign method to suppress the damaging
399 effects of α -toxin and may be of benefit during joint irrigation for septic arthritis caused by *S.*
400 *aureus*.

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403 *Staphylococcus aureus* strain 8325-4.

References

1. Kaandorp CJ, Dinant HJ, van de Laar MA, Moens HJ, Prins AP, Dijkmans BA. Incidence and sources of native and prosthetic joint infection: a community based prospective survey. *Ann. Rheum. Dis.* 1987;56(8):470-475.
2. Goldenberg DL. Septic arthritis. *Lancet* 1998;351:197-202.
3. Kaandorp CJ, Krijnen P, Moens HJ, Habbema JD, van Schaardenburg D. The outcome of bacterial arthritis: a prospective community-based study. *Arth. Rheum.* 1997;40:884-892.
4. Dubost JJ, Soubrier M, De Champs C, Ristori JM, Bussiere JL, Sauvezie B. No changes in the distribution of organisms responsible for septic arthritis over a 20 year period. *Ann. Rheum. Dis.* 2002;61(3):267-269.
5. Mathews CJ, Weston VC, Jones A, Field M, Coakley G. Bacterial septic arthritis in adults. *Lancet* 2010;375:846-855.
6. Krieg AM. A possible cause of joint destruction in septic arthritis. *Arth. Res. Ther.* 1999;1:3-4.
7. Cunningham R, Cockayne A, Humphreys H. Clinical and molecular aspects of the pathogenesis of *Staphylococcus aureus* bone and joint infections. *J. Med. Microbiol.* 1996;44:157-64.
8. O'Callaghan RJ, Callegan MC, Moreau JM, Green LC, Foster TJ, Hartford OM, et al. Specific roles of alpha-toxin and beta-toxin during *Staphylococcus aureus* corneal infection. *Infect. Immun.* 1997;65(5):1571-1578.
9. Schmitz FJ, Veldkamp KE, Van Kessel KP, Verhoef J, Van Strijp. Delta-toxin from *Staphylococcus aureus* as a co-stimulator of human neutrophil oxidative burst. *J. Infect. Dis.* 1997;176(6):1531-1537.
10. Dajcs JJ, Thibodeaux BA, Girgis DO, O'Callaghan RJ. Corneal virulence of *Staphylococcus aureus* in an experimental model of keratitis. *DNA Cell Biol.* 2002;21(5-6):375-382.
11. Hayashida A, Bartlett AH, Foster TJ, Park PW. *Staphylococcus aureus* beta-toxin induces lung injury through syndecan-1. *Amer. J. Pathol.* 2009;174(2):509-518. doi: 10.2353/ajpath.2009.080394.
12. Smith IDM, Winstanley JP, Milto KM, Doherty CJ, Czarniak E, Amyes SGB, et al. Rapid chondrocyte death induced by *Staphylococcus aureus* toxins in a bovine cartilage explant model of septic arthritis. *Osteoarth. Cart.* 2013;21(11):1755-65.
13. Clement R, Hall AC, Howie SEM, Simpson AHRW. A murine model of septic arthritis demonstrates that infection with an alpha toxin-producing strain of *S. aureus* leads to significantly elevated levels of chondrocyte death within 48 hours of infection when compared with infection with an alpha toxin-deficient mutant strain. *Bone Joint J.* 2016;98-B (SUPP 16):20.
14. Smith IDM, Milto KM, Doherty CJ, Amyes SGB, Simpson AHRW, Hall AC. A potential key role of alpha-haemolysin of *Staphylococcus aureus* in mediating chondrocyte death in septic arthritis. *Bone Jt. Res.* 2018; IN PRESS.
15. Hultgren OH, Stenson M, Tarkowski A. Role of IL-12 in *Staphylococcus aureus* triggered arthritis and sepsis. *Arthritis Res* 2001;3:41-47.
16. Hwang SG, Yu SS, Poo H, Chun JS. C-Jun/Activator protein-1 mediates interleukin-1-induced dedifferentiation but not cyclooxygenase-2 expression in articular chondrocytes. *J. Biol. Chem.* 2005;280:29780-29787.

17. Guo Y, Li J, Hagström E, Ny T. Protective effects of plasmin(ogen) in a mouse model of *Staphylococcus aureus* induced arthritis. *Arthritis Rheum.* 2008;58:764–772.
18. Hong EH, Yun HS, Kim J, Um HD, Lee KH, Kang CM, *et al.* Nicotinamide phosphoribosyltransferase is essential for interleukin-1-mediated dedifferentiation of articular chondrocytes *via* SIRT1 and extracellular signal-regulated kinase (ERK) complex signaling. *J. Biol. Chem.* 2011;286:28619–28631.
19. Chubinskaya S, Cs-Szabo G, Kuettner KE. ADAM-10 message is expressed in human articular cartilage. *J. Histochem. Cytochem.* 1998;46(6):723-729.
20. Chubinskaya S, Mikhail R, Deutsch A, Tindal MH. ADAM-10 protein is present in human articular cartilage primarily in the membrane-bound form and is upregulated in osteoarthritis and in response to IL-1 α in bovine nasal cartilage. *J. Histochem. Cytochem.* 2001;49:1165-1176.
21. Wilke GA, Bubeck Wardenburg J. Role of a disintegrin and metalloprotease 10 in *Staphylococcus aureus* alpha-hemolysin-mediated cellular injury. *Proc. Natl. Acad. Sci. USA* 2010;107(30):13473-13478.
22. Bhakdi S, Tranum-Jensen J. Alpha-toxin of *Staphylococcus aureus*. *Microbiol. Rev.* 1991;55(4):733-751.
23. Vandenesch F, Lina G, Henry T. *Staphylococcus aureus* hemolysins, bi-component leukocidins and cytolytic peptides: a redundant arsenal of membrane-damaging virulence factors? *Front. Cell. Infect. Microbiol.* 2012;16:2:12. doi: 10.3389/fcimb.2012.00012.
24. Cooper LZ, Madoff MA, Weinstein L. Hemolysis of rabbit erythrocytes by purified Staphylococcal alpha-toxin. I. Kinetics of the lytic reaction. *J. Bacteriol.* 1964;87:127-35.
25. Baumgarten M, Bloebaum RD, Ross SD, Campbell P, Sarmiento A. Normal human synovial fluid. *J. Bone Jt. Surg.* 1985;67(9):1336-1339.
26. Amin AK, Huntley JS, Simpson AHRW, Hall AC. Increasing the osmolarity of joint irrigation solutions may avoid injury to cartilage: A pilot study. *Clin. Orthop. Rel. Res.* 2010;468(3):875-884.
27. Amin AK, Simpson AHRW, Hall AC. Iatrogenic articular cartilage injury: the elephant in the orthopaedic operating theatre. The surgeon's role in chondroprotection. *Bone Joint J. (Editorial).* 2017;99-B:1555-6.
28. Dinges MM, Orwin PM, Schlievert PM. Exotoxins of *Staphylococcus aureus*. *Clin. Microbiol. Rev.* 2000;13(1):16-34.
29. Gamba G. Molecular physiology and pathophysiology of electroneutral cation chloride cotransporters. *Physiol. Rev.* 2005;85:423-493.
30. Reichwein J, Hugo F, Roth M, Sinner A, Bhakdi S. Quantitative analysis of the binding and oligomerization of staphylococcal alpha-toxin in target erythrocyte membranes. *Infect. Immun.* 1987;55(12):2940-2944.
31. Freer J. Cytolytic toxins and surface activity. *Toxicon* 1982;20(1):217-221.
32. Reddi B. Why Is Saline So Acidic (and Does It Really Matter?). *Int. J. Med. Sci.* 2013;10(6):747-750.
33. Harshman S, Boquet P, Duflot E, Alouf JE, Montecucco C, Papini E. Staphylococcal α -toxin: A study of membrane penetration and pore formation. *J. Biol. Chem.* 1989;264(25):14978-14984.

519
520 34. Bortoleto R, Ward R. A stability transition at mildly acidic pH in the alpha-hemolysin (alpha-
521 toxin) from *Staphylococcus aureus*. *FEBS Letters* 1999;459(3):438-442.
522
523 35. Harshman S, Sugg N. (1985). Effect of calcium ions on staphylococcal alpha-toxin-induced
524 hemolysis of rabbit erythrocytes. *Infect. Immun.* 1985;47(1):37-40.
525
526 36. Xie Y, Yang L. Calcium and magnesium ions are membrane-active against stationary-phase
527 *Staphylococcus aureus* with high specificity. *Scientific Reports* 2016;6(1):20628:1-8
528 doi:10.1038/srep20628.
529
530 37. Li Z, Liu D, Ding J, Gong L, Wang Y, Tang P, *et al.* Effects of cold irrigation on early results
531 after total knee arthroplasty: a randomised, double-blind controlled study. *Medicine* (Baltimore).
532 2016;95(24):e3563. doi: 10.1097/MD.0000000000003563.
533
534 38. Chan PF, Foster SJ. The role of environmental factors in the regulation of virulence-
535 determinant expression of *Staphylococcus aureus* 8325-4. *Microbiology* 1998;144:2469-2479.
536
537 39. Bush PG, Hall, AC. The osmotic sensitivity of isolated and *in situ* bovine articular
538 chondrocytes. *J. Orthop. Res.* 2001;19(5), 768-778.
539
540 40. DeLeo FR, Chambers HF. Re-emergence of antibiotic-resistant *Staphylococcus aureus* in the
541 genomics era. *J. Clin. Invest.* 2009;119:2464-2474.
542
543 41. Los FC, Randis TM, Aroian RV, Ratner AJ. Role of pore-forming toxins in bacterial infectious
544 diseases. *Microbiol. Mol. Biol. Rev.* 2013;77(2):173-207 doi: 10.1128/MMBR.00052-12.
545
546 42. Balaban N, Collins LV, Cullor JS, Hume EB, Medina-Acosta E, Viera da Motta O, *et al.*
547 Prevention of diseases caused by *Staphylococcus aureus* using the peptide RIP. *Peptides*
548 2000;21:1301-1311.
549
550 43. Foletti D, Strop P, Shaughnessy L, Hasa-Moreno A, Casas MG, Russell M, *et al.* Mechanism of
551 action and *in vivo* efficacy of a human-derived antibody against *Staphylococcus aureus* α -
552 hemolysin. *J. Mol. Biol.* 2013;425(10):1641-54. doi: 10.1016/j.jmb.2013.02.008.
553
554 44. Weeks AC, Balzli CL, Caballero A, Tang A, O'Callaghan R. Identification and potency of
555 cyclodextrin-lipid inhibitors of *Staphylococcus aureus* alpha-toxin. *Curr. Eye Res.* 2012;37:87-93.
556
557 45. Hu C-M J, Fang RH, Luk BT, Zhang L. Nanoparticle-detained toxins for safe and effective
558 vaccination. *Nat. Nanotechnol.* 2013;8(12):933-938. doi:10.1038/nnano.2013.254.

Figures

Figure 1. Raised osmolarity reduced rabbit erythrocyte haemolysis induced by *S. aureus* α -toxin. The % haemolysis (%H) was measured over 60mins under either (A) chronic or (B) acute challenge with various osmolarities as follows; 0.9% saline (control, 300mOsm (●)) and saline osmolarity raised by sucrose addition to 400mOsm (□), 600mOsm (▲) or 900mOsm (inverted triangle) either before toxin addition (chronic) or at approx. 20-30% haemolysis (indicated by the bar and arrow – ‘osmolarity change’). The panels labelled (ii) show the % haemolysis data at t=60mins plotted as a function of osmolarity, and the panels labelled (iii) show the rate of change of % haemolysis/10mins plotted as a function of osmolarity. In this and subsequent figures, significant differences are indicated as follows: * $P<0.05$; ** $P<0.01$; *** $P<0.001$). Results are means \pm s.e.m. from (5(2)).

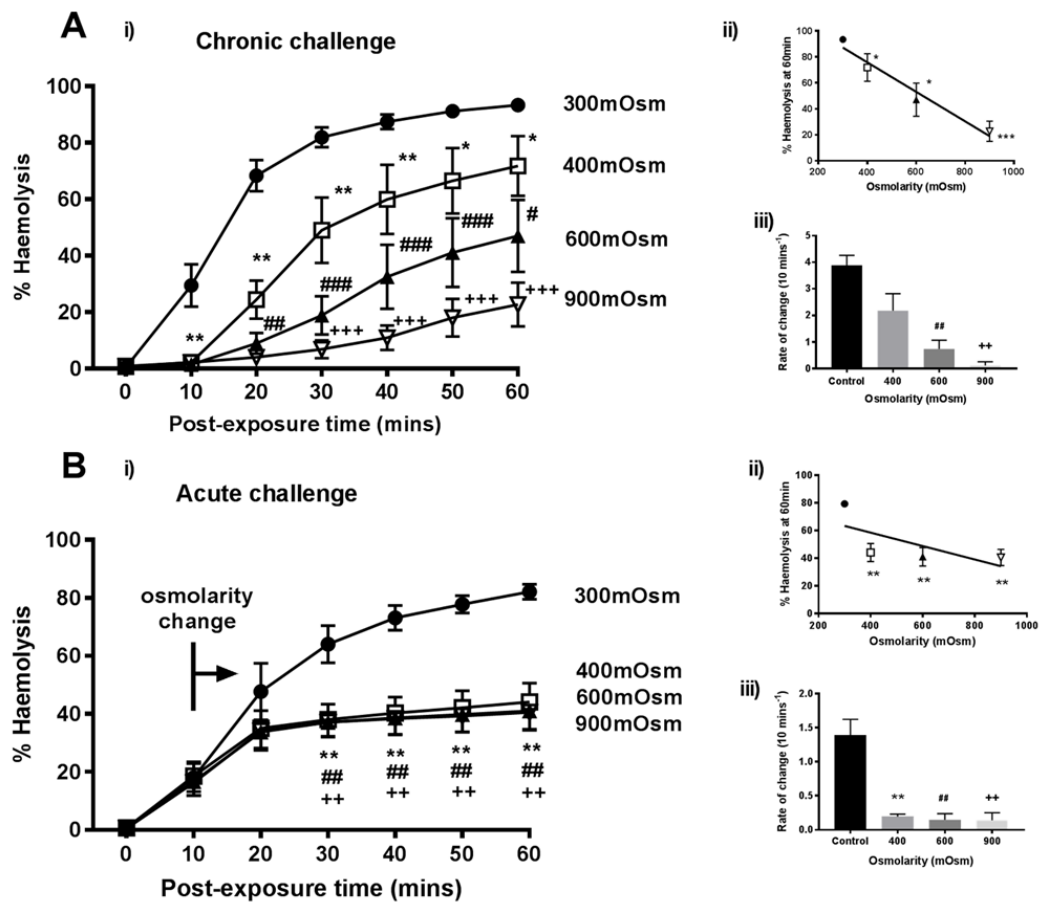
Figure 2. Decreasing temperature suppressed rabbit erythrocyte haemolysis induced by *S. aureus* α -toxin. The % haemolysis (%H) was measured over 60mins under either (A) chronic or (B) acute challenge at different temperatures as follows; 37°C (control) (●), 25°C (□), 4°C (▲) either before toxin addition (chronic) or at about 20% haemolysis (indicated by the bar and arrow – ‘temperature change’). The panels labelled (ii) show the % haemolysis data at t=60mins plotted as a function of temperature, and the panels labelled (iii) show the rate of change of % haemolysis/10mins plotted as a function of temperature. Results are means \pm s.e.m. from (4(2)).

Figure 3. Divalent cations inhibited rabbit erythrocyte haemolysis induced by *S. aureus* α -toxin. The % haemolysis (%H) was measured over 60mins under either (A) chronic or (B) acute challenge in the presence of Ca^{2+} or Mg^{2+} normal saline (NS; 0.9% NaCl control) (●), Ca^{2+} (5mM) or Mg^{2+} (5mM) (▲) either before toxin addition (chronic) or at about 20% haemolysis (indicated by the bar and arrow – divalent challenge). The panels labelled (ii) show the % haemolysis data at t=60mins plotted as a function of temperature, and the panels labelled (iii) show the rate of change of % haemolysis/10mins plotted as a function of temperature. Results are means \pm s.e.m. from (4(2)).

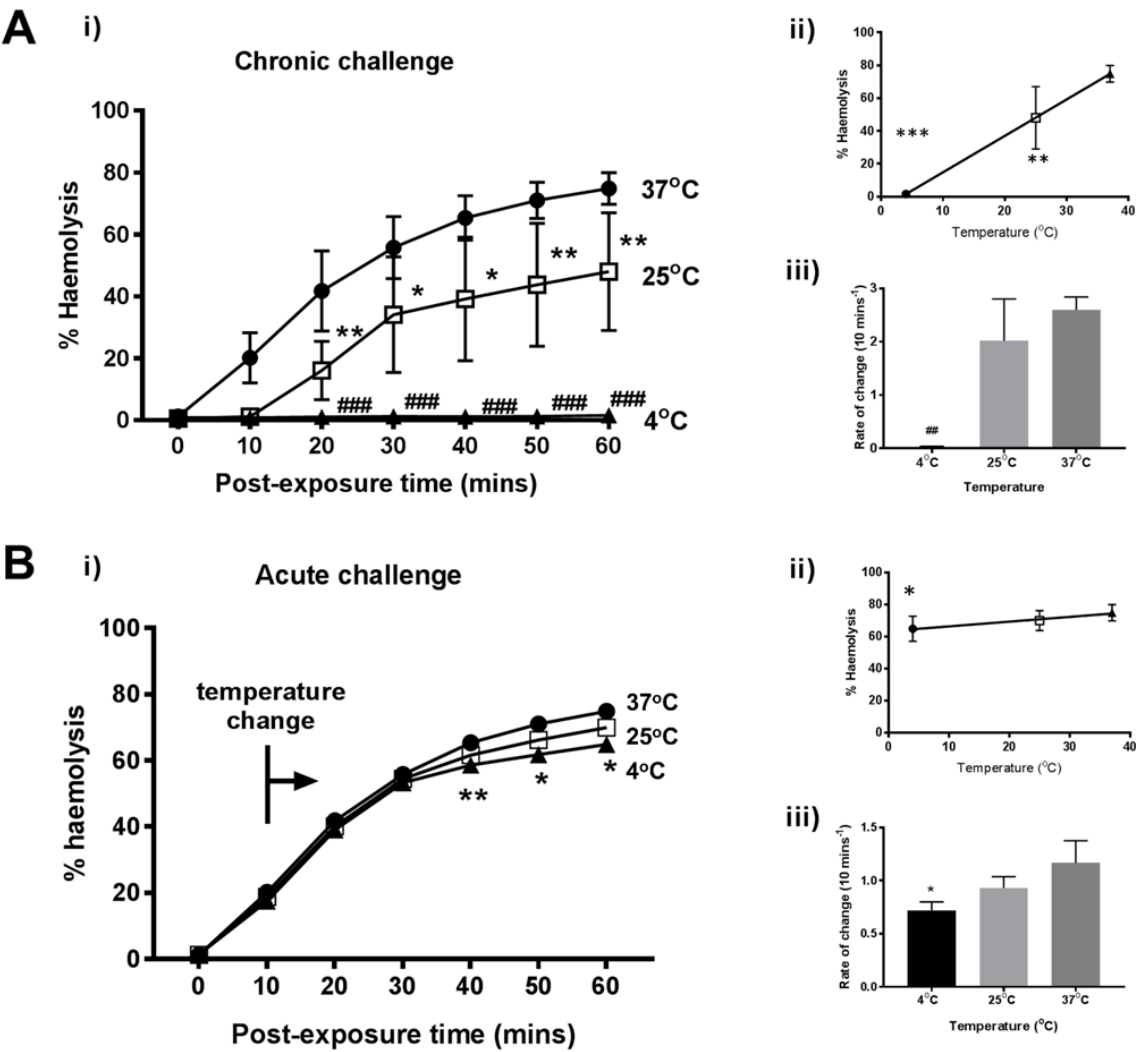
Figure. 4. Effect of varying pH on rabbit erythrocyte haemolysis induced by *S. aureus* α -toxin. The % haemolysis (%H) was measured over 60mins under chronic challenge at pH values of 7.2 (control) (□), 6.5 (●), and 8.0 (▲) in HBSS containing the buffer HEPES (10mM) with pH altered using HCl or NaOH (all at 300mOsm). The panel labelled (ii) shows the % haemolysis data at t=60mins plotted as a function of temperature, and the panel labelled (iii) shows the rate of change of % haemolysis/10mins plotted as a function of temperature. Results are means \pm s.e.m. from (3(2)).

597 Figure 5. **The protective effect of optimised saline on rabbit erythrocyte haemolysis induced**
598 **by *S. aureus* α -toxin.** The % haemolysis (%H) was measured over 60mins under either (A)
599 chronic or (B) acute challenge in the presence normal saline (NS; 0.9% NaCl control) (●), HBSS
600 (□), or optimised saline (OS; 0.9% NaCl, 900mOsm, 5mM MgCl₂, 37°C) (▲) either before toxin
601 addition (chronic) or at about 20% haemolysis (indicated by the bar and arrow – challenge). The
602 panels labelled (ii) show the rate of change of % haemolysis/10mins for chronic and acute
603 challenges respectively in the various solutions. Results are means \pm s.e.m. from (5(2)).
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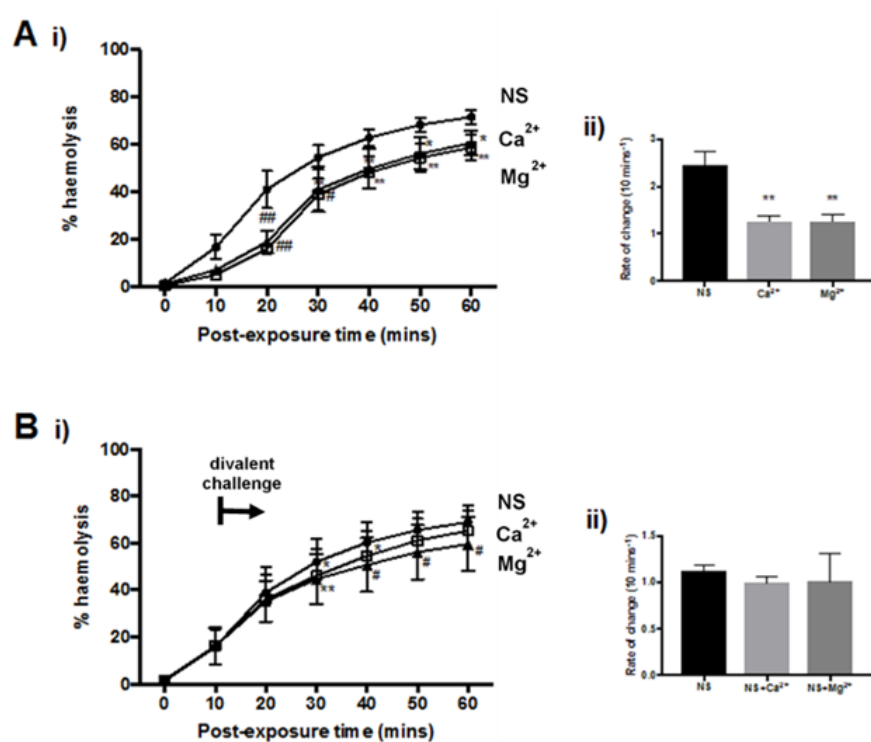
Liu & Hall, 2018 Figure 1.



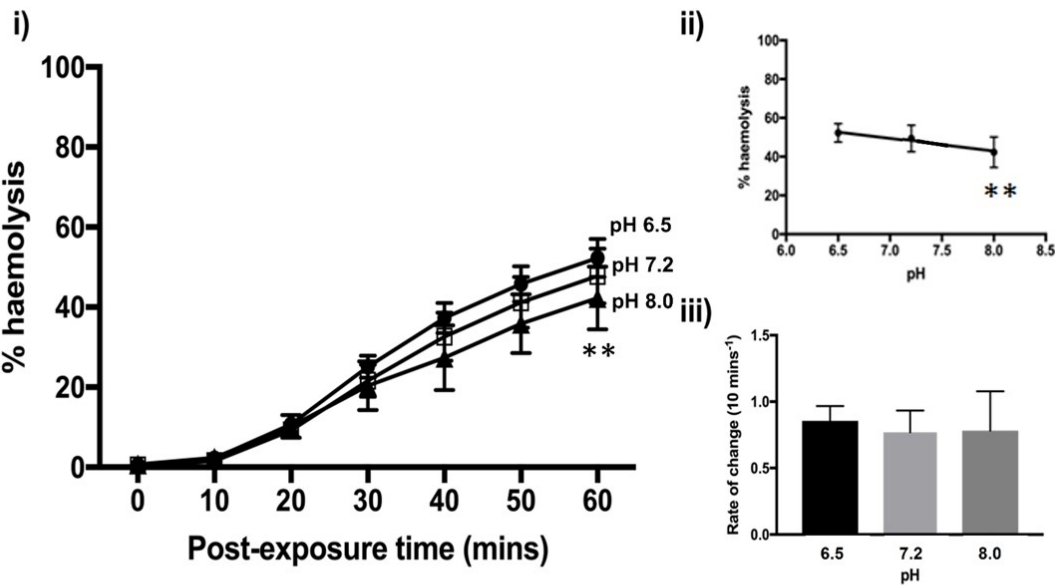
Liu & Hall, 2018 Figure 2.



Liu & Hall, 2018 Figure 3.



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Liu & Hall, 2018 Figure 5.

